Developmental Biology **216,** 57–71 (1999)
Article ID dbio.1999.9469, available online at http://www.idealibrary.com on IDEAL®

Expression with Implications for Patterning of the Precardiac Field

Yongmei Jiang,* Thomas A. Drysdale,† and Todd Evans*,[1]

**Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461; and †Lawson Research Institute, St. Joseph's Health Centre, Departments of Paediatrics, Physiology, and Zoology, University of Western Ontario, London, Ontario, Canada*

Interactions between the key regulatory genes of the cardiogenic pathway, including those from the GATA and Nkx2 transcription factor families, are not well defined. Treating neurula-stage *Xenopus* embryos with retinoic acid (RA) causes a specific block in cardiomyocyte development that correlates with a progressive reduction in the region of the presumptive heart-forming region expressing Nkx2.5. In contrast, RA does not block expression of the GATA-4/5/6 genes, which are transcribed normally in an overlapping pattern with Nkx2.5 throughout cardiogenesis. Instead, GATA-4/5/6 transcription levels are increased, including an expansion of the expression domain corresponding to lateral plate mesoderm that is part of the early heart field, but that normally is progressively restricted in its ability to contribute to the myocardium. GATA-dependent regulatory sequences of the Nkx2.5 gene that implicate GATA-4/5/6 as upstream positive regulators were described recently. However, our experiments also indicate that GATA factors might normally antagonize transcription of Nkx2.5. To test this hypothesis we generated a dominant negative isoform of GATA-4 (SRG4) capable of inhibiting transcription of GATA-dependent target genes. Ectopic expression of SRG4 results in a transient expansion of the Nkx2.5 transcript pattern, indicating that a normal function of GATA factors is to limit the boundary of the Nkx2.5 expression domain to the most anterior ventral region of the heart field. Regulatory mechanisms altered by excess RA must function normally to limit GATA-4/5/6 expression levels, to define the region of Nkx2.5 expression and regulate myocardial differentiation.

Key Words: **GATA factors; tinman; cardiogenesis; *Xenopus;* embryogenesis.**

INTRODUCTION

The vertebrate heart develops from bilateral heart-forming regions of lateral plate mesoderm, believed to be specified before or during gastrulation (for review, see Lyons, 1996; Mohun and Sparrow, 1997). Experiments in *Xenopus* determined that signals from the organizer (Sater and Jacobson, 1990b) and deep endoderm (Nascone and Mercola, 1995) induce lateral mesoderm adjacent to Spemann's organizer to become heart. The exact nature of this inductive signaling event is not known, although bone morphogenetic proteins appear to play a significant role in initiating the cardiogenic program (Schultheiss *et al.*, 1997). Genes from two transcription factor families are expressed during early development in overlapping patterns within mesoderm and associated anterior endoderm of the presumptive heart-forming region. One set of factors includes GATA-4/5/6 of the GATA family of zinc finger-containing transcription factors (Jiang and Evans, 1996; Kelley *et al.*, 1993; Laverriere *et al.*, 1994) and the other is the Nkx2 (Csx) family of homeobox-containing transcription factors (Evans *et al.*, 1995; Newman and Krieg, 1998; Tanaka *et al.*, 1998; Tonissen *et al.*, 1994) related to the *Drosophila* heart-specification gene called tinman (Bodmer, 1993).

The early coexpression of Nkx2 and GATA factors in the cardiac crescent, prior to expression of known cardiomyocyte differentiation genes, is consistent with a model in

[1] To whom correspondence should be addressed at 1300 Morris Park Avenue, Chanin 503, Bronx, NY 10461. Fax: (718) 430-8988. E-mail: tevans@aecom.yu.edu.

0012-1606/99 $30.00

which members of both families cooperate in specifying or otherwise regulating the development of cardiogenic progenitors (Charron and Nemer, 1999; Evans, 1997). Both subfamilies are implicated in regulating the expression of multiple cardiac-specific genes (Grepin *et al.*, 1994; Ip *et al.*, 1994; Lyons *et al.*, 1995; Molkentin *et al.*, 1994) and may do so by physical interaction within a common protein complex (Durocher *et al.*, 1997; Durocher and Nemer, 1998; Sepulveda *et al.*, 1998). The importance of Nkx2 and GATA factors in heart development has been tested by loss-of-function experiments. Expression of dominant-interfering isoforms of Nkx2 proteins results in a complete block to *Xenopus* heart development (Fu *et al.*, 1998; Grow and Krieg, 1998). However, gene disruption experiments in mice have not demonstrated cell-specification functions for individual Nkx2 or GATA family members, but are instead consistent with critical roles in heart tube morphogenesis. For example, disruption of Nkx2.5 leads to a nonlooping heart tube phenotype (Lyons *et al.*, 1995; Tanaka *et al.*, 1999), and mutations in the human gene are identified as causing congenital heart disease (Schott *et al.*, 1998). In the GATA-4 null mutant mouse there is a failure of the two heart primordia to fuse at the ventral midline, although cardiac differentiation in the twinned bifid hearts that develop appears normal (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). The primary abnormality of these mice is likely to be defective endoderm development, leading by an unknown mechanism to failure in fusion of the two heart primordia (Narita *et al.*, 1997). Some less obvious aspects of cardiomyocyte differentiation may also be affected by the Nkx2.5 and GATA-4 mutations, and additional roles in cardiomyocyte specification may be compensated by other family members.

In comparison to the Nkx2 family, for which there is evidence of function in cardiomyocyte development (Cleaver *et al.*, 1996), the genetic role for GATA factors during cardiogenesis appears relatively complex. GATA-4 has been demonstrated to have a positive effect on the expression of cardiac differentiation genes (Grepin *et al.*, 1997), so that it would appear that different GATA factors can have some nonoverlapping functions. Overexpression of GATA-6 in *Xenopus* embryos was observed to block the differentiation of heart precursors (Gove *et al.*, 1997), suggesting that a decline in GATA-6 expression levels may be a cue for cardiomyocyte differentiation. In mice, GATA-6 is required for extraembryonic development and thus mice lacking this gene die before a potential role in cardiac differentiation can be assessed (Koutsourakis *et al.*, 1999; Morrisey *et al.*, 1998), although GATA-6 mutant ES cells are able to contribute to the heart in chimeric embryos. However, using antisense oligonucleotides, it is necessary to deplete transcripts encoding each of the three chick genes to detect cardiac abnormalities, indicating that all three GATA factors act in a common morphogenetic pathway (Jiang *et al.*, 1998). Therefore, GATA factors may be important for endoderm and mesoderm development related to cardiomyocyte proliferation and differentiation, as well as heart tube formation and morphogenesis.

Retinoids regulate an important signaling pathway for cardiogenesis, and either an excess or a deficit of retinoic acid (RA) is disruptive to normal heart development. Similar cardiogenic defects are caused by a lack of embryonic retinoids (Zile, 1998) or if retinoid signaling is blocked through targeted disruption of retinoid receptor genes (Gruber *et al.*, 1996; Kastner *et al.*, 1994; Lee *et al.*, 1997; Luo *et al.*, 1996; Sucov *et al.*, 1994). Retinoids may also be important for the diversification of atrial and ventricular tissues and in the regulation of chamber-specific gene expression (Yutzey *et al.*, 1994, 1995). The GATA-4 gene fails to be expressed in vitamin A deficient (VAD) quail embryos that develop an abnormal nonlooping heart tube, while Nkx2.5 expression levels are not grossly affected (Kostetskii *et al.*, 1999). These experiments link a requirement for retinoids in establishment of a specific GATA-dependent cardiac transcriptional program associated with posterior heart tube development and tube morphogenesis. In *Xenopus*, addition of RA blocks cardiac differentiation, even if applied after expression patterns for Nkx2 and GATA factors are established (Drysdale *et al.*, 1997). Transcript levels for Nkx2.5, Nkx2.3 (Drysdale *et al.*, 1997), and Nkx2.9 (P. A. Krieg, personal communication) decline rapidly in the heart-forming region in response to RA exposure. The decline in transcripts encoding tinman-related genes may be responsible for the block to cardiac differentiation. Alternatively, retinoic acid may be acting upstream of the Nkx2 genes on a more general cardiac development program. In this system RA is unlikely to be affecting cell specification because *Xenopus* heart specification is completed by the end of gastrulation (Sater and Jacobson, 1989), while RA can affect heart differentiation of mid-neurula-stage embryos.

Because the expression patterns of the Nkx2 and GATA factors are largely overlapping during early cardiogenesis, it is feasible that they might regulate the expression of each other; indeed the mouse promoter for Nkx2.5 has binding sites for GATA factors and itself (Lien *et al.*, 1999; Searcy *et al.*, 1998). There is evidence from the analysis of mouse mutations that cross-talk can occur between the GATA-4 and the GATA-6 genes; meanwhile, Nkx2.5 is not required for normal expression of GATA-4 (Tanaka *et al.*, 1999). In the *Xenopus* model, using retinoic acid to block expression of Nkx2 expression and heart development allows us to test if GATA factors are dependent on Nkx2 expression or are coregulated by a common or linked signaling pathway. We find that GATA factors are not dependent on Nkx2.5 for expression, nor are GATAs sufficient for expression of Nkx2.5 or for cardiac differentiation. Ectopic expression of a dominant negative GATA-4 confirms that GATA factors may normally restrict the expression domain of Nkx2.5 and that down-regulation of GATAs during neurula stages might be an important component of the normal cardiogenic program.

MATERIALS AND METHODS

Embryo and Explant Culture

Mature *Xenopus* females were injected with 700–800 IU of human chorionic gonadotrophin (Sigma) and the ovulated eggs were fertilized *in vitro* using minced testis. Embryos were dejellied with 2.5% cysteine, pH 8.0, and cultured in 20% Steinberg's solution or 0.1× MBS. Embryos were staged according to Nieuwkoop and Faber (1967). Explants were isolated using fine forceps and cultured in modified Danilchik's medium (Peng, 1991) without BSA. Culture dishes were coated with a thin layer of 1% agarose.

Retinoic Acid Treatment

Retinoic acid (all-*trans;* Sigma) was prepared as a 1 mM stock solution in dimethyl sulfoxide (DMSO). Embryos were immersed in 1 μM retinoic acid at various embryonic stages and unless stated the treatment was continuous. Control embryos were treated with 1 μl/ml DMSO.

RNA Analysis

Whole-mount *in situ* hybridization was performed as described (Jiang and Evans, 1996). The *Xenopus* GATA-4/5/6 cDNA clones used to generate the probes are also described (Jiang and Evans, 1996) and the xNkx2.5 probe is described in Tonissen *et al.* (1994). The TnIc probe was prepared as described (Drysdale *et al.*, 1994). The xSox17α probe was a gift from the H. R. Woodland lab (Hudson *et al.*, 1997) and prepared following *Sma*I digestion using T7 polymerase. In some experiments, embryos were postfixed, washed in water, dehydrated, washed twice in xylenes, and then infused with paraplast overnight. After embedding, paraplast blocks were trimmed and 10-μ sections cut and floated onto "Superfrost Plus" microscope slides (Fisher), dried overnight, dewaxed in xylenes, and coverslipped using Permount (Fisher). Semiquantitative RT/PCR to measure transcript levels of GATA-4 and EF-1α was performed as described (Jiang and Evans, 1996) and quantified by phoshorimaging following gel electrophoresis.

Dominant Negative xGATA-4 (SRG4)

A region of the xGATA-4 cDNA encompassing both zinc fingers and the entire DNA-binding domain (from amino acid 147 to 304) was isolated by PCR using primers that incorporated a stop codon and permitted in-frame fusion at the N-terminus with sequences encoding the strong repression domain (amino acids 1–69) of the mouse Mxi1 gene (Schreiber-Agus *et al.*, 1995). The Mxi1 cDNA was a gift from the R. DePinho laboratory. The chimeric cDNA encoding a protein called SRG4 was confirmed by sequencing and then subcloned into vector pCDNA3, for both *in vitro* transcription/translation and transfection experiments in tissue-culture cells, and into vector pGEMHE (providing globin gene untranslated sequences) for *in vitro* transcription to generate capped RNA for microinjection. For control experiments, a near-identical cDNA was generated (mtSRG4) containing a single mutation of the SR domain (Mxi-SR-pro; a proline for leucine substitution at amino acid residue 19 (Schreiber-Agus *et al.*, 1995)) that abolishes the ability of SR to interact with sin3 or repress transcription. The SRG4 protein was tested for activity using *in vitro*-generated rabbit reticulocyte lysates (Promega) by gel mobility-shift experiments as described (Yang and Evans, 1992). Cotransfection experiments into QT6 fibroblasts were performed and analyzed as described (Evans and Felsenfeld, 1991; Gao *et al.*, 1998), using the GATA-4, SRG4, and mtSRG4 expression plasmids and the αD3-luc and pCH110 β-galactosidase reporter plasmids. The αD3-luc reporter is identical to that described (Evans and Felsenfeld, 1991) except that the CAT gene is replaced with a luciferase coding sequence derived from pGL3basic (Promega). Each transfection contained 2 μg reporter, a total of 4 μg of expression vectors, and 0.25 μg internal control reporter. For microinjection experiments, capped mRNA was prepared *in vitro* from linearized pGEMHE/SRG4 using a kit from Ambion. To determine the left/right embryonic axis, fertilized eggs were tipped in medium containing 7% Ficoll and marked on the presumptive dorsal side using Nile blue dye as described (Peng, 1991). At the eight-cell stage, a single left dorsal/vegetal blastomere was injected with 4.5 nl of solution containing 250 pg SRG4 RNA (or in control samples, mtSRG4 RNA) in water containing 5 mg/ml fluorescein-dextran (Molecular Probes; D-1820), to facilitate lineage tracing.

RESULTS

GATA-4/5/6 Transcription Is Not Inhibited in RA-Treated Embryos That Fail to Express Cardiomyocyte Markers

It was shown previously that treatment of *Xenopus* embryos with 1 μM RA at stage 20 blocks differentiation of the heart and causes a rapid loss of Nkx2.5 and Nkx2.3 transcripts in the presumptive heart region. In contrast to myocardial markers, endocardial development is apparently resistant to this treatment, indicated by persistent expression of the X-msr gene (Drysdale *et al.*, 1997). A superficially normal heart tube forms that fails to loop or form any beating tissue. The loss of expression in the tinman-related genes might be sufficient to explain the subsequent failure to express markers of myocardial differentiation at later stages, or it might reflect a more general loss of the cardiovascular gene expression program. In the latter case, a loss of GATA-4/5/6 expression in the RA-treated embryos might be predicted, given the overlapping expression patterns of these two gene families throughout early cardiovascular development.

As shown in Fig. 1a, treatment of embryos at stage 20 with 1 μM RA results in a near-complete loss of Nkx2.5 and cardiac troponin I (TnIc) transcripts. The loss is complete in many embryos, but the examples shown here indicate the position of the presumptive heart and illustrate an important point. Treatment of embryos with RA does not diminish equally transcript levels throughout the heart-forming region. Rather, the spatial domain of transcripts is progressively deleted toward the ventral midline, so that in many cases only a small region of ventral tissue expresses Nkx2.5 and a myocardial differentiation program. Note that Nkx2.5 transcripts are detected readily in pharyngeal endoderm, an anterior tissue that is not a normal expression domain for GATA factors. In surprising contrast, we find that all three GATA family members are expressed in the RA-treated embryos and the domains of expression are even

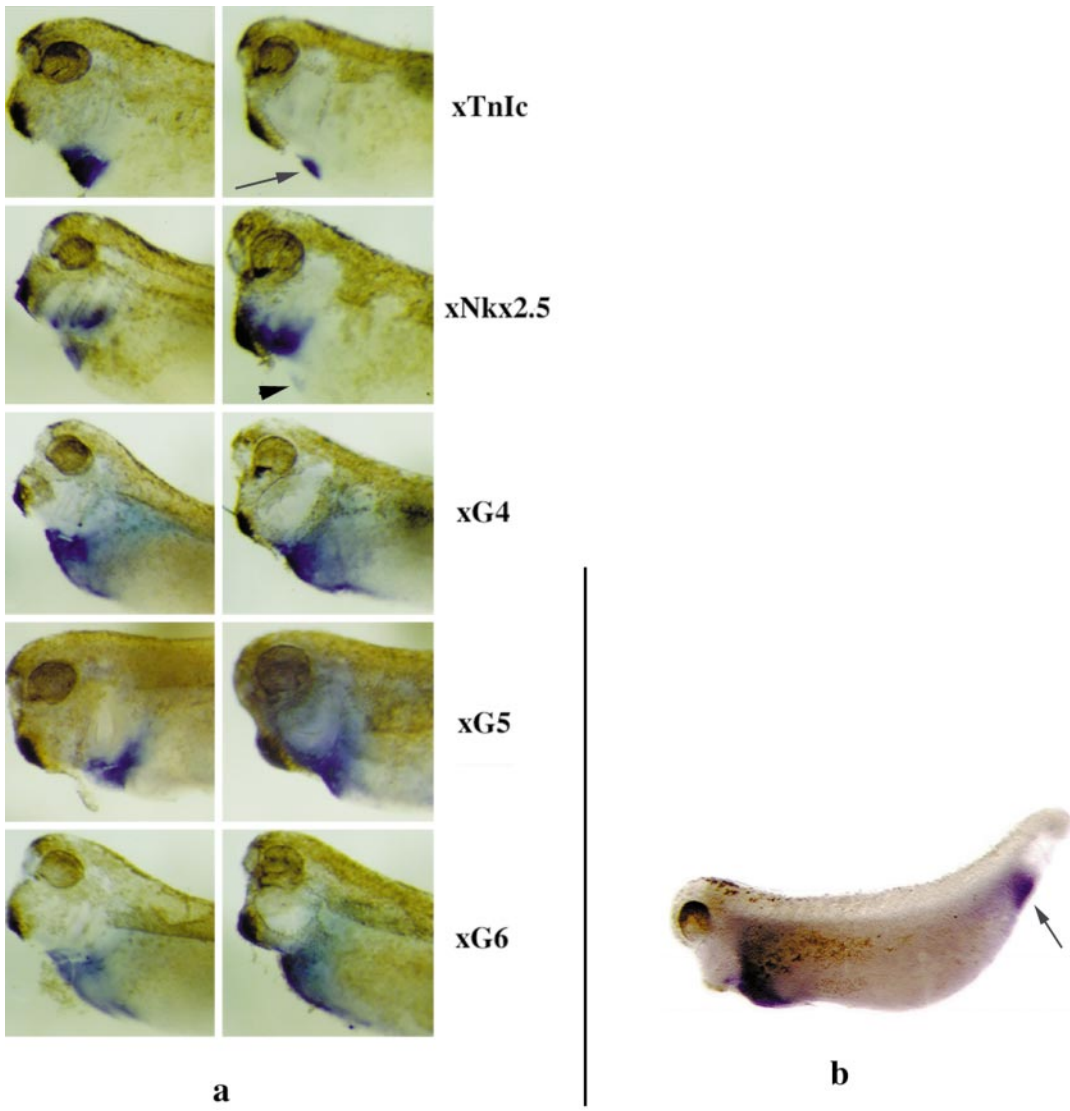
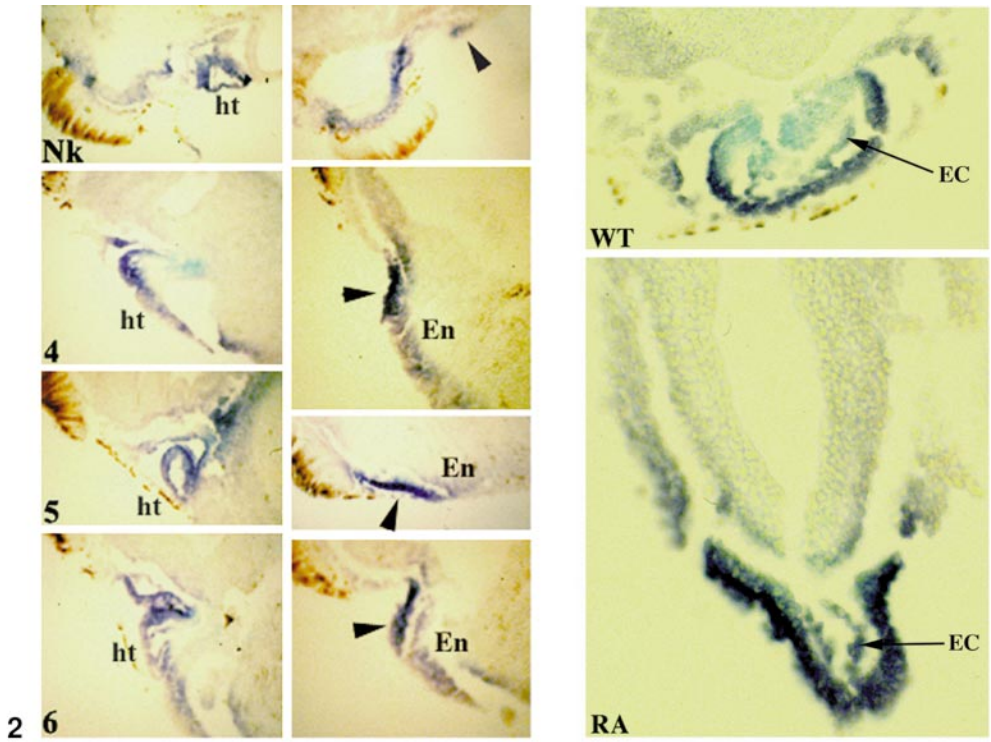
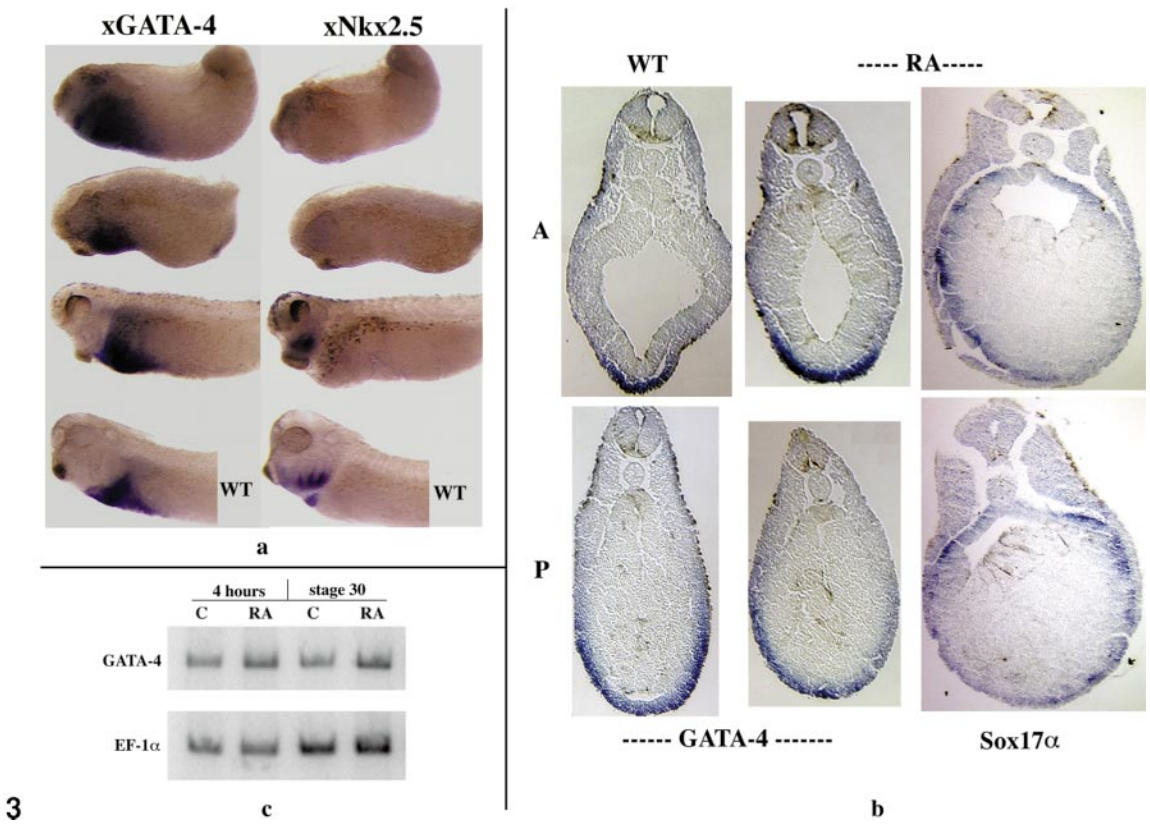


FIG. 1. GATA-4/5/6 transcription is not inhibited by RA treatment, which blocks myocardial differentiation. (a) Whole-mount *in situ* hybridization was performed using probes specific to transcripts for cardiac troponin I, Nkx2.5, or GATA-4/5/6, as indicated on the right. Embryos are oriented with anterior to the left, dorsal to the top. RA was added at stage 23 and embryos were allowed to develop until stage 35. Examples of control embryos, treated with DMSO alone, are shown on the left; examples of RA-treated embryos are on the right. The arrow and arrowheads in the two upper right images indicate the small patch of differentiated myocardium that remains in these embryos, at the most ventral position of the presumptive heart-forming region. Note that the pharyngeal endoderm, which does not express GATA-4/5/6, remains positive for Nkx2.5 following RA treatment. The Nkx2.5 levels appear increased in pharyngeal endoderm of this RA-treated embryo due to extended staining in order to observe the weak signal in the residual heart region. However, enhanced levels of Nkx2.5 RNA are not observed generally in pharyngeal endoderm of RA-treated embryos (Drysdale *et al.*, 1997). (b) An example of an RA-treated embryo analyzed for xGATA-6 RNA to illustrate the induction of a posterior domain of transcription (arrow). A similar activation is seen for GATA-5 but not for GATA-4. Our previous work implicated GATA-6 in the regulation of progenitor cells of the gut (Gao *et al.*, 1998), and so this domain is likely to correspond to the presumptive hindgut.

somewhat expanded. The patterns, as detected by whole-mount *in situ* hybridization, are similar but not identical for each of the three genes. In RA-treated embryos, the patterns extend more broadly dorsal and caudal relative to control embryos. The expanded domain that encompasses the GATA-4/5/6 pattern does not correspond in any obvious manner to a specific tissue. For both GATA-5 and GATA-6, there was in addition ectopic expression of transcripts detected around the developing hindgut (Fig. 1b).





RA-Treated Embryos Maintain both Mesodermal and Endodermal Expression of GATA Factors

GATA-4/5/6 are expressed normally in both the precardiac mesoderm and the associated endoderm of the presumptive anterior foregut. Therefore, the expression of GATA factors in RA-treated embryos might reflect a relative expansion of the endodermal expression domain relative to the presumptive myocardium. To characterize further the transcript patterns for xGATA-4/5/6, RA-treated and control embryos were sectioned following whole-mount *in situ* hybridization. As shown in Fig. 2a (sagittal sections), transcripts of xGATA-4/5/6 persist in the unfolded layer of tissue that would normally have formed differentiated myocardium and the endocardium of the RA-treated embryos. Expression in the persisting endocardial layer as well as the closely associated anterior endoderm is more easily analyzed in cross section, as shown for example with GATA-4 in Fig. 2b. Similar patterns were found for GATA-5 and GATA-6 (not shown).

Early Exposure to RA Further Enhances GATA-4 Expression in the Complete Absence of Anterior Structures

When retinoic acid is applied to *Xenopus* embryos at the end of neurulation, it affects specifically the developing heart region while the rest of the embryo is relatively normal. Although there is no detectable myocardial-specific gene expression, there is limited cardiac morphogenesis (Drysdale *et al.*, 1997). When retinoic acid is applied prior to gastrulation, there is a general loss of all anterior structures including heart (Durston *et al.*, 1989; Sive *et al.*, 1990). This complicates interpreting a direct relationship between the addition of retinoic acid at early stages and cardiac development, but does allow us to determine whether the persistent expression of GATA factors is dependent on the initial presence of a cardiogenic program.

We examined embryos exposed continuously to 1 μM RA starting at gastrulation (stage 10.5) or at midneurula (stage 14/15) and compared development and expression patterns with control embryos and those treated at stage 20. In embryos treated early we obtained the expected axis defects and general loss of anterior structures. In those embryos, the expression of GATA-4/5/6 genes, particularly GATA-4, is not only detected at the anterior ventral region, but also expanded to the dorsoanterior boundary (Fig. 3a), while Nkx2.5 transcripts are not detected. Examination of sections from the embryos reveal that the GATA transcripts are present in the presumptive mesoderm and the associated endoderm cells, similar to the pattern in control embryos but enhanced. This is particularly obvious in the case of GATA-4, as shown in Fig. 3b. We used a probe to the endoderm marker Sox17α (Hudson *et al.*, 1997) to demonstrate that endoderm and mesoderm are both still present in the RA-treated embryos and that GATA-4 transcripts persist clearly in the mesoderm layer (Fig. 3b). To further confirm that RA treatment results in an increase in the transcript levels of GATA-4, embryos were exposed to continuous RA treatment from stage 10.5, either for 4 h or to stage 30; RNA was isolated and assayed for GATA-4 transcripts by semiquantitative RT/PCR. In either case, the levels of GATA-4 transcripts are increased twofold in the RA-treated embryos relative to the control embryos (Fig. 3c).

A Dominant Negative Form of xGATA-4 Can Inhibit the Activation of GATA Target Genes

Our observation that expression levels of xGATA-4/5/6 genes are increased by RA treatment while the Nkx2.5 gene is coordinately inactivated leads to the unexpected possi-

FIG. 2. GATA-4/5/6 transcription is maintained and enhanced in both the mesoderm and the endoderm of RA-treated embryos. (a) Sagittal sections taken from embryos analyzed first by whole-mount *in situ* hybridization for Nkx2.5 or GATA-4/5/6 (top to bottom). Embryos are oriented as in Fig. 1; the cement gland is visible as brown tissue (adjacent to the Nk in the upper left image). Sections from control DMSO-treated embryos are shown on the left; samples from RA-treated embryos are on the right. The position of the folded heart (ht) is indicated on the left. The myocardial (arrowhead) and associated endoderm layer (En), both of which are positive for GATA-4/5/6, are indicated on the right. (b) Cross section of a similarly treated embryo analyzed for GATA-4 transcripts. Dorsal is to the top. The endocardial (EC) layer is indicated for both control (top) and RA-treated (lower) samples.

FIG. 3. Earlier treatment of embryos with RA results in further expansion of the GATA-4 transcript pattern. (a) Embryos were analyzed (and are oriented) as in Fig. 1a (except that the embryos were fixed at stage 30) for GATA-4 (left) or Nkx2.5 (right) transcripts following continuous treatment of RA added at stage 10.5 (top), stage 14 (2nd from top), or stage 20 (3rd from top). Embryos on the bottom were controls treated only with DMSO. Even in embryos with anterior truncations the GATA-4 gene is transcribed in an expanded region of the lateral plate. GATA-5 and GATA-6 were also transcribed in these embryos, but did not show such an obvious expansion of the pattern. (b) Cross sections are shown of embryos treated with RA beginning at stage 10.5 and analyzed for GATA-4 transcripts as in (a). Compared to the control embryos (left), the GATA-4 transcript pattern is enhanced and expanded in the RA-treated embryos (middle). To confirm the identity of the endoderm layer, embryos were also analyzed for transcripts from the Sox17α gene. This pattern was unaffected by RA treatment (not shown). Sections were taken either from an anterior (A) position (top) or from a more posterior (P) position caudal to the heart proper (bottom). (c) Embryos were treated with RA from stage 10.5 and RNA was harvested from embryos after 4 h (left lanes) or at the equivalent of stage 30 (right lanes) and analyzed for GATA-4 or control EF-1α transcript levels by semiquantitative RT/PCR. Consistent with the signals detected by *in situ* hybridization, the steady-state transcript levels for GATA-4 were increased 100% by RA treatment.

bility that GATA genes and the Nkx2.5 gene might have some functions in cardiogenesis that are antagonistic. To further investigate the genetic relationship between GATA genes and Nkx2.5, a dominant negative form of GATA-4 was generated (SRG4, Fig. 4a) by fusing the "strong repression" (SR) domain of the murine Mxi1 protein to the amino-terminus of a peptide encompassing the DNA-binding domain from GATA-4 (G4). The SR domain of Mxi1 interacts with the general chromatin repressor protein sin3 to mediate suppression of myc oncogenic activity (Schreiber-Agus *et al.*, 1995). It has been shown that when fused to a heterologous DNA-binding domain, the SR domain can target sin3 to gene-specific binding sites leading to repression (Harper *et al.*, 1996). A mutant form of SRG4 (mtSRG4) was also generated, containing a single amino acid alteration of the SR domain that abolishes interaction with sin3 and is unable to repress transcription.

The SRG4 (and the mtSRG4; not shown) bind specifically to a consensus GATA-binding *cis*-element (Fig. 4b). The ability of the SRG4 construct to suppress transcription directed by GATA-4/5/6 was tested in a cell culture system. In this study, each of the three cardiac GATAs *trans*-activates a model GATA-dependent reporter gene approximately eightfold (an example is shown for GATA-4 in Fig. 4c). Coexpression of SRG4 in the transfection assay completely suppresses the *trans*-activation, even below the basal level. In the experiment shown, the SRG4 expression vector is in threefold excess over the GATA-4 expression vector, but suppression to basal level is found even using equal ratios (not shown). Coexpressed SRG4 was equally capable of suppressing activation of the reporter by GATA-5 and GATA-6 (not shown). In contrast, the control mtSRG4 was unable to inhibit activation by wild-type GATA-4 (Fig. 4c). Similar results were found for a protein consisting of the DNA-binding domain of GATA-4 alone; if expressed in further excess this protein or the mtSRG4 protein could inhibit coexpressed wild-type GATA-4 activity only about twofold (not shown). Therefore, SRG4 can function as a dominant negative isoform by competing with wild-type GATA factors to inhibit activation of GATA-dependent targets.

Expression of SRG4 Expands the Posterior Expression Domain of the Nkx2.5 Gene during Early Stages of Cardiogenesis

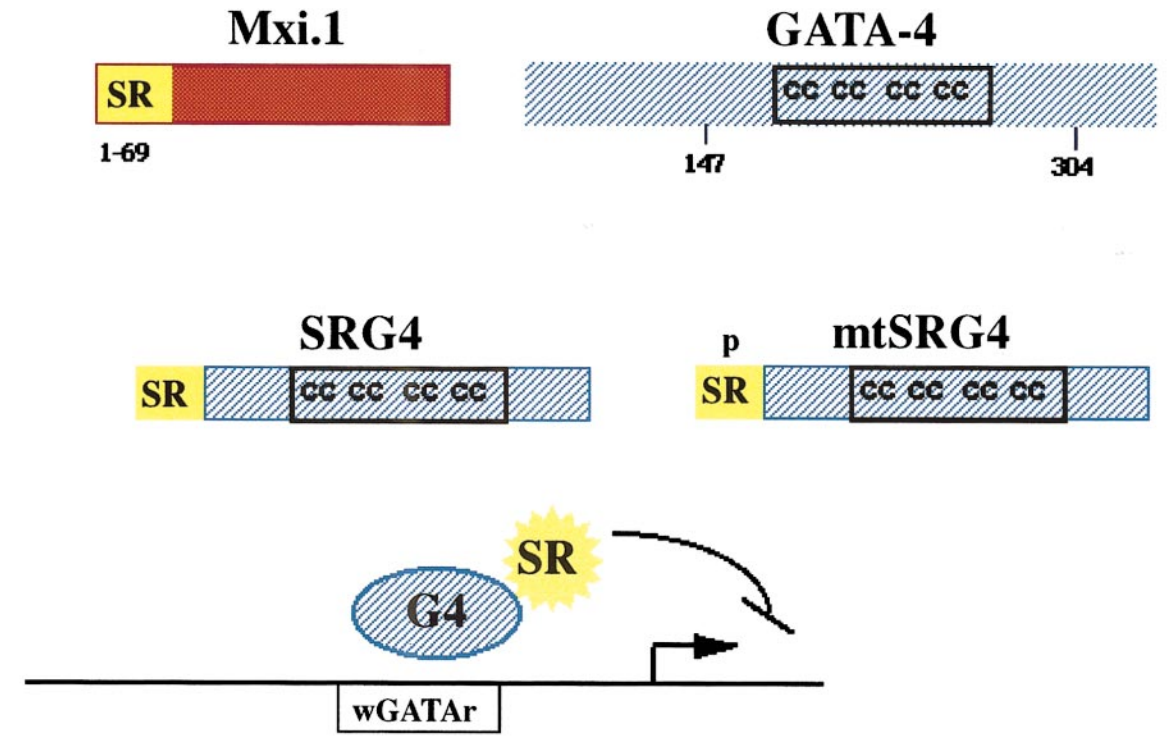
Our data using exogenous RA treatment correlated increased expression of GATA factors with inhibition of Nkx2.5. If this represents a genetic interaction, a prediction can be made that decreasing GATA factor activity might result in enhanced expression of Nkx2.5. To inhibit GATA-dependent gene expression *in vivo*, *in vitro*-transcribed SRG4 mRNA was injected into a left dorsal–vegetal blastomere of eight-cell-stage embryos. According to the fate map (Moody, 1987), the dorsal–vegetal blastomeres contribute to the future heart. In control experiments, RNA encoding mtSRG4 was injected. The injected embryos were assayed by whole-mount *in situ* hybridization at stage 26/27 for the transcription of Nkx2.5, just prior to fusion of the two bilateral primordia at the ventral midline. As shown in Fig. 5a, the Nkx2.5 transcript pattern is seen in control (lacZ injected) embryos in symmetric patches of presumptive heart myocardium, as well as pharyngeal endoderm. In 42% of the embryos injected with SRG4 RNA, an asymmetric Nkx2.5 transcription pattern was detected (64/152, compared to 2% of the mtSRG4-injected embryos; see Table 1). This expanded domain of expression was not seen when embryos were injected with other RNAs, including those encoding full-length GATA-4. Of the embryos with asymmetric expression (estimated visually as having one side roughly twice or more the area of the other), the expanded pattern was detected in 80% of the cases on the presumed injected (left) side. Analysis of co-injected lineage tracer confirmed that the accuracy of targeting RNA to the left dorsal–vegetal blastomere was 75%. The expanded domain of Nkx2.5 transcripts is restricted to mesoderm and is located primarily in a domain more posterior to that found in normal embryos (Fig. 5b).

The embryos injected with either SRG4 or mtSRG4 generally went on to develop without obvious abnormalities. The expression of SRG4 is for technical reasons transient and is presumably overcome by normal expression of GATA factors in the developing cardiovascular region. It is not surprising that the hearts of SRG4 embryos are normal, given the regulative ability of *Xenopus* embryos to control heart size. We attempted to increase the amount of SRG4 by targeting both sides of the developing heart. Unfortunately, injection into both dorsal/vegetal blastomeres at the eight-cell stage results in severe phenotypic abnormalities in a high percentage of embryos that complicated further analysis of cardiogenesis (short, stubby embryos displaying an expanded "pigeon chest," but with no consistent heart phenotype). We conclude that inhibition of GATA-dependent gene expression leads to ectopic Nkx2.5 transcription, consistent with a normal function for GATA factors in regulating the Nkx2.5 expression domain.

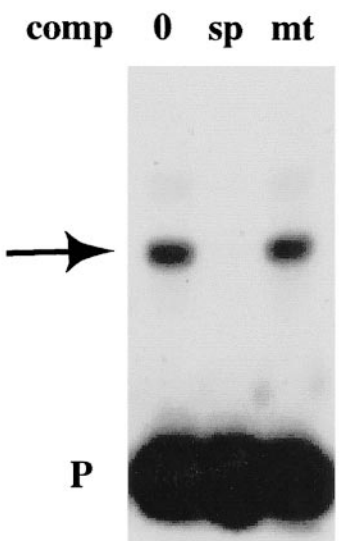
DISCUSSION

GATA-4/5/6 Expression Is Enhanced in Response to Retinoic Acid, Concurrent with Decreased Transcription of Nkx2.5 and a Failure in Cardiomyocyte Differentiation

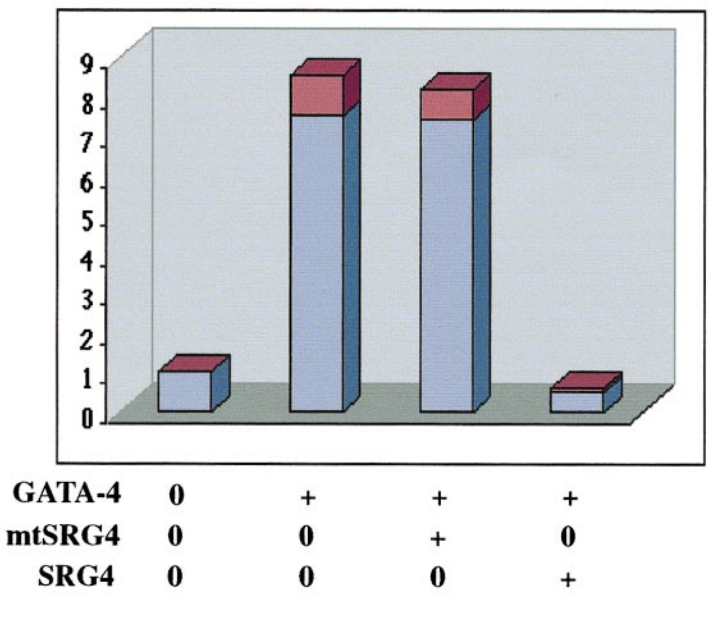
The phenotype of the neurula-stage RA-treated embryo is a strikingly specific defect in Nkx2 gene expression and a block to myocardial differentiation. Given the overlapping coexpression of GATA and Nkx2 family members throughout early stages of heart development, it might be predicted that these embryos would have a similar defect in GATA factor expression. However, transcription of GATA-family members is activated, rather than inhibited, by retinoid signaling (Arceci *et al.*, 1993; Boylan *et al.*, 1995; Kostetskii



a



b



c

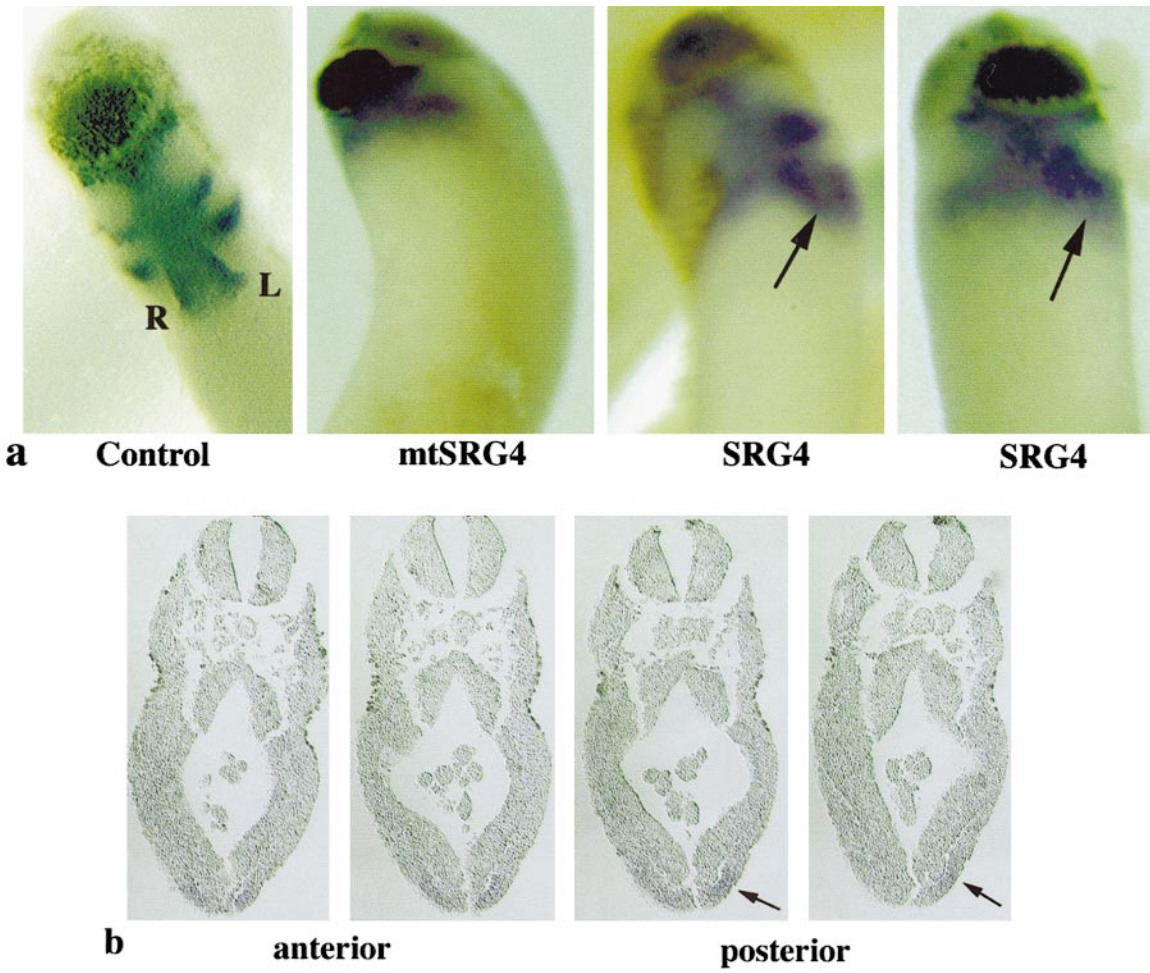


FIG. 5. Ectopic targeted expression of SRG4 results in an expanded pattern of Nkx2.5 transcription. Examples are shown of embryos injected at the 8-cell stage into one left-side dorsal–vegetal blastomere with RNA encoding control LacZ RNA, mtSRG4, or SRG4, as indicated. Embryos were fixed at stage 26 and analyzed by whole-mount *in situ* hybridization for Nkx2.5 transcripts; processed embryos are viewed from the ventral side, anterior is up. The control embryo is slightly older so the right (R) and left (L) sides of the primitive heart tube are fusing at the midline. Compared to the symmetric pattern seen here or in the prefusing primordia of the mtSRG4-injected embryo, the SRG4-injected embryos display an expanded domain of Nkx2.5 transcription in the left primordium (arrows). (b) An embryo injected in the left blastomere with SRG4 RNA was processed as in (a), and sections were taken through the region of the fusing primordia. Shown are four sections taken from progressively more posterior regions (left to right). Note that in the most anterior regions the Nkx2.5 pattern is not significantly changed, but left-side enhanced transcripts are detected in more posterior sections (arrows).

FIG. 4. SRG4 functions as a dominant negative isoform capable of inhibiting activation of GATA-4 targets. (a) Schematic illustration of the domain structure of SRG4. The strong repression (SR) domain of Mxi.1 is fused to a region of GATA-4, containing both zinc fingers (cc cc), that constitutes the DNA-binding and cofactor interaction domains of GATA-4. A control isoform (mtSRG4) was also generated, containing a single amino acid change (leucine to proline, p) that abolishes the ability of SR to repress gene expression. SRG4 is predicted to repress GATA-dependent target genes through interaction with normal GATA-binding sites containing a WGATAR consensus sequence. (b) A gel mobility-shift experiment demonstrates that SRG4 binds with specificity to a consensus GATA *cis*-element. The GATA-binding probe (P) was end-labeled and incubated with rabbit reticulocyte lysate charged with *in vitro*-generated RNA encoding SRG4. The reaction included no competitor (0) or a 50-fold molar excess of unlabeled oligomer containing the same specific (sp) binding site or a similar oligomer containing a mutation (mt) of the GATA motif. (c) A well-characterized GATA-dependent reporter (αD3-luc) was cotransfected into QT6 fibroblasts with (+) or without (0) a GATA-4 expression vector, along with an empty vector (to maintain total amount of vector DNA), mtSRG4, or SRG4 as indicated. The level of activation relative to the basal promoter activity is shown after normalizing for activity of a cotransfected β-galactosidase reporter that is not regulated by GATA-4 expression. The results shown are from three independent experiments, each performed in duplicate; red stacked bars represent standard deviation. SRG4 was equally capable of repressing activation by xGATA-5 or xGATA-6 (not shown).

TABLE 1

Injected mRNA	Total no. embryos injected	Left > right	Right > left
SRG4 mRNA	152	51 (33.6%)	13 (8.6%)
mtSRG4 RNA	106	2 (1.9%)	0

Note. Embryos were injected at the 8-cell stage in a single left dorsal–vegetal blastomere with 250 pg of RNA encoding either SRG4 or mtSRG4. Embryos were fixed at stage 26 and analyzed for Nkx2.5 transcripts as shown in Fig. 5. Left > right indicates embryos with an expanded domain of Nkx2.5 on the left heart primodium, prior to fusion; 2 such embryos are shown for example in Fig. 5a. To determine the accuracy of targeting, 244 embryos were analyzed by fluorescence microscopy before being fixed for the co-injected dextran tracer. In this case, 183 (75%) were FITC-positive primarily on the left side. The other embryos were injected mistakenly on the right side, or the analysis was complicated by cell mixing. The lineage tracing results indicate that the 13 SRG4-injected embryos showing a right > left pattern can be explained by mistargeting of the injected RNA.

et al., 1999; Soudais *et al.*, 1995). Regulation of retinoid signaling is a conserved component of normal cardiogenesis and excess RA present during pregnancy or administered directly to embryos or precardiac fields causes severe cardiac abnormalities, including cardia bifida (Hoffman and Eichele, 1994; Smith and Dickman, 1997). Although many studies have documented changes in cardiomyocyte development in the presence of excess retinoids, these results must be interpreted with caution because it is not yet established that the phenotypes are related mechanistically to normal development. In our studies regarding the influence of exogenous RA on the regulation of Nkx2.5 and GATA factors, we feel that the results are likely to be of relevance for several reasons:

First, the phenotype is specific to the developing heart in embryos that appear otherwise quite normal, so the effect is unlikely to be derived from an indirect teratogenic response, for example, as seen when early treated embryos show a general loss of anterior structures, including the heart. The phenotype is seen when embryos are treated at the neurula stage, long after specification and axis patterning is thought to be finished. Second, the phenotype is consistent (and opposite) to that seen in mice with targeted disruptions in retinoic acid receptor genes (Kastner *et al.*, 1997). These mutant mice display defective cardiac morphogenesis with a thin myocardium attributed to precocious ventricular cardiomyocyte differentiation. Therefore, a lack of retinoid signaling accelerates differentiation, while in our studies excess retinoids block differentiation. Finally, and most importantly, the expression patterns for GATA-4 and Nkx2.5 following RA treatment are exactly what would be predicted based on our analysis of the vitamin A-deficient quail embryo, completely lacking in embryonic retinoid signaling. This embryo, which has a differentiated myocardium but a severe heart tube morphogenetic defect, fails to express GATA-4, while Nkx2.5 transcripts are still present. Therefore, in the absence of RA the GATA-4 gene is off and the Nkx2.5 is on, while in the presence of excess RA the GATA-4 transcript pattern is enhanced and the Nkx2.5 gene is turned off. Retinoic-responsive elements in the Nkx2.5 gene are not described. Our data implicate GATA factors as an intermediary and are consistent with a normal function for RA in regulating the embryonic expression pattern of GATA factors with relevance to the developing cardiovascular system.

GATA Factors May Function Upstream of Nkx2, but Are Not Sufficient in the Absence of Nkx2 Expression to Direct Cardiomyocyte Development

Transcripts for GATA-4/5/6 can be detected faintly as early as stage 3 in gastrulating cells of the chick primitive streak that include the cardiogenic progenitors. Although it is not known if functional protein is expressed this early, by the cardiac crescent stage transcripts for all three genes are detected readily in largely overlapping patterns throughout a broad region of lateral plate mesoderm and associated endoderm, in cells that eventually generate (among other things) endocardium, myocardium, great vessel endothelia, and foregut endoderm. This early pattern of GATA factor expression therefore marks cells that contribute generally to the cardiovascular system, while part of the Nkx2.5 pattern is overlapping, within those progenitors that will form the myocardium. Our results suggest that the observed changes in GATA expression may play a role in the loss of Nkx2.5 expression in the presumptive heart region. As the exogenous RA is able to block the differentiation of the heart, it would suggest that GATA factors alone are unable to allow cardiac differentiation and thus implicate Nkx2 family members as playing an essential role in this process. There is no direct evidence from mouse mutational studies that GATA factors regulate Nkx2 family members or vice versa. However, these analyses are complicated by likely functional redundancy among family members. Indirect evidence that GATA factors function upstream of and regulate the expression of Nkx2.5 comes from transgenic mouse experiments that determined functionally relevant enhancer sequences of the Nkx2.5 gene that contain GATA binding sites (Lien *et al.*, 1999; Searcy *et al.*, 1998). These experiments indicate that activation of the Nkx2.5 gene in presumptive cardiomyocytes is dependent on GATA factors. If this were a direct epistatic relationship we would predict that expression of a dominant negative GATA-4 isoform might reduce Nkx2.5 transcript levels. Instead the opposite result was obtained; the pattern of Nkx2.5 was expanded in a significant fraction of the embryos, particularly along the ventral/caudal midline, in cells that do not normally contribute to the myocardium.

Although this result may appear counterintuitive, it is entirely consistent with the expression of Nkx2.5 when GATA factor activity is affected by alterations in retinoid signaling. Therefore, increased expression of GATA factors in the RA-treated *Xenopus* embryos correlates with decreased transcription of Nkx2.5. Similarly, depletion of GATA factors in the VAD embryo does not lead to a lack of Nkx2.5. The transcript levels for Nkx2.5 in the VAD quail were not quantified, but we predict that they might even be enhanced in the GATA-4-deregulated embryos. In this case an argument for functional compensation of Nkx2.5 expression by GATA-6 is unlikely, because GATA-6 transcripts are also decreased in the VAD embryo, albeit less dramatically relative to GATA-4. A simple hypothesis that is consistent with our data in RA-treated frog embryos, the VAD quail embryo, the SRG4-injected frog embryos, and the transgenic mouse experiments is that GATA factors regulate the expression of Nkx2.5 but can do so in either a positive or a negative manner, depending on the cell context. Therefore, in presumptive cardiomyocytes, GATA factors might activate or maintain the expression of Nkx2.5, while in regions of the embryo posterior and lateral to the myocardial progenitors (for example, in the region of the developing inflow tract) GATA factors might inhibit the Nkx2.5 gene. This differential regulation by GATA factors could be important either in defining the myocardial subpopulation or in patterning of the developing heart tube along the anterior/posterior axis. Currently, the mechanism for differential regulation of Nkx2.5 can only be speculated, but could involve cell- or region-specific cofactors different levels of specific GATA factors, or posttranslational modification of specific GATA factors. Perhaps most interesting, the GATA cofactor FOG-2 mediates either activation or repression of GATA-dependent promoters, depending on the gene and the cell type tested (Lu *et al.*, 1999; Svensson *et al.*, 1999). This is precisely the type of mechanism that could lead to differential regulation of Nkx2.5 by GATA factors in the developing heart.

A Potential Role for GATA Factors in Regulating the Boundaries of the Precardiac Field

The concept of a heart morphogenetic field refers to that region of the embryo that is capable of contributing to the developing heart, including progenitors that do not normally do so. The presence and boundary of a field can be determined experimentally in explant assays (that presumably relieve some cells of the field from negative regulatory mechanisms that normally restrict cardiac differentiation) or in cell-ablation experiments (in which additional cells of the field are redirected to a heart cell fate by unknown regulatory mechanisms to compensate for those that are lost). For example, explant assays that scored for the presence of beating heart tissue showed that in *Xenopus* neurula embryos (stage 20) the area that can form heart tissue includes anterior ventral and ventrolateral mesoderm (Sater and Jacobson, 1990a). By stage 28 the field is restricted to the anterior ventral mesoderm. The molecular basis of how a heart field is restricted has until recently been elusive. However, we note that the expanded domain of GATA factor expression seen in stage 20 RA-treated embryos corresponds at least in part to the lateral mesoderm that is initially part of the heart field but normally loses potency. GATA factors may therefore represent molecular markers for the initial heart field, which becomes blocked from further development in the RA-treated embryos. Indeed, recent cell-ablation experiments in zebrafish support the hypothesis that GATA-4 expression delineates the boundary of the heart field. In zebrafish, only the anterior half of the Nkx2.5-positive lateral plate mesoderm contributes to the cardiogenic progenitor population. When cells of the normal cardiogenic region are ablated, more anterior cells that express GATA-4 acquire a cardiac cell fate (Serbedzija *et al.*, 1998).

Our data support a model in which the pattern of GATA factor expression helps determine the boundary of the cardiac field and the levels are critical to regulating the normal progression to a differentiated heart. GATA factors might be required for the activation or maintenance of an Nkx2-dependent cardiac differentiation program, but the levels and boundaries must also be controlled to allow restriction of the heart field and permit terminal differentiation. Therefore (see Fig. 6), overexpression of GATA-6 leads to a reduction in the potential of cells to express a differentiated cardiomyocyte program (Gove *et al.*, 1997). According to this model (Fig. 6, middle), it might be predicted that overexpression of wild-type GATA-4 would lead to a reduction in the expression domain of Nkx2.5. However, we did not find this, nor were changes in the Nkx2.5 transcript patterns detected by GATA-6 overexpression in the study by Gove *et al.* (1997). Therefore, GATA factors themselves may not be limiting in the control of Nkx2.5 and probably do not function alone in this capacity, but downstream of and/or in parallel to other genes regulated by RA. In contrast, repression of GATA-dependent target genes (using the dominant negative isoform SRG4) leads to an expansion of the Nkx2.5 transcript pattern, indicating that one or more GATA target genes function normally in defining the Nkx2.5 pattern and restricting those cells of the cardiac field that are competent for myocardial development. The dominant negative isoform may interfere with pathways for each of the GATA-4/5/6 factors, resulting in a transient depletion of some factor(s) needed for proper heart patterning. Depletion of any single GATA factor might not be sufficient to generate a similar result. We note that aspects of this mechanism may be relevant only in vertebrate species that have a regulative heart field (Ehrman and Yutzey, 1999).

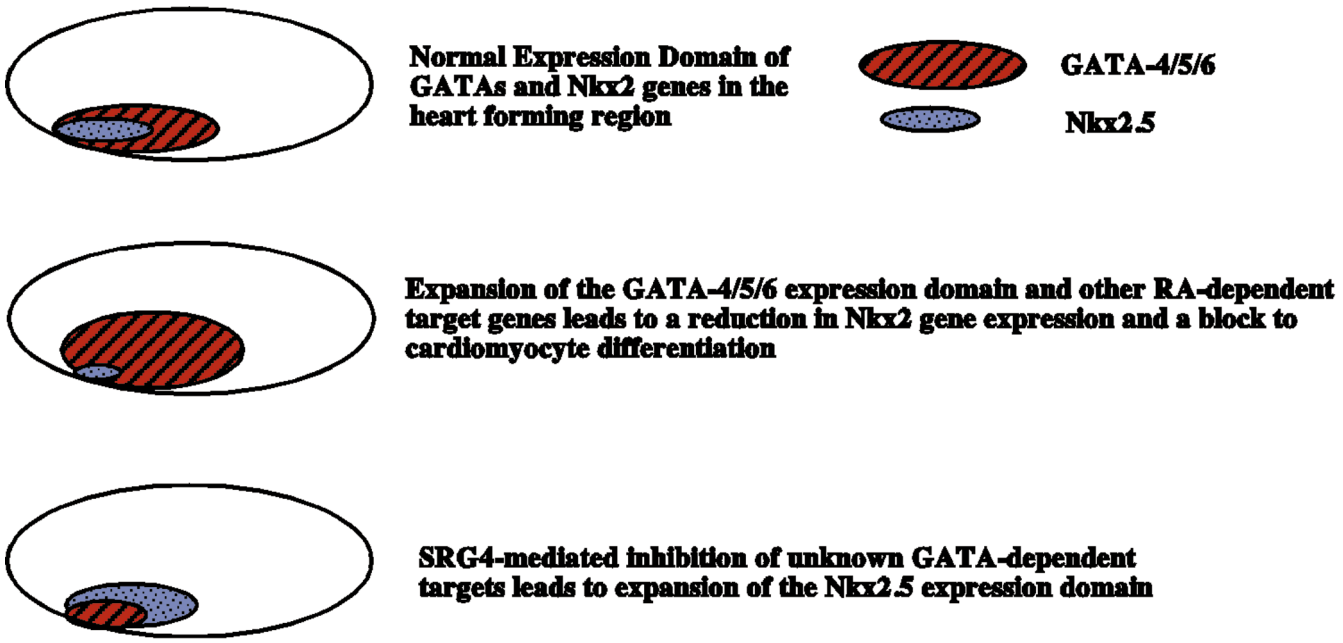


FIG. 6. Based on the data presented, we hypothesize that the levels or boundaries of GATA-4/5/6 expression delineate the region of the heart field that will express Nkx2 genes and be competent for cardiomyocyte development. When GATA factors are overexpressed, cardiomyocyte differentiation can be repressed. Inhibition of GATA-dependent gene expression results in an expanded pattern of Nkx2.5 transcripts, perhaps providing competence to a larger part of the heart field to contribute to the cardiomyocyte population. The model is consistent with additional reports in the literature demonstrating the ability of enhanced levels of GATA-6 (Gove *et al.*, 1997) or Nkx2.5 (Cleaver *et al.*, 1996) to inhibit or expand, respectively, the population of differentiated cardiomyocytes (although note that this is a relatively late effect and neither GATA-4 or GATA-6 overexpression alone is reported to alter Nkx2.5 transcript patterns). The domain of Nkx2 expression in the pharyngeal endoderm is not indicated in the schematic, although we note that RA does not activate GATA expression in the pharyngeal endoderm and also does inhibit Nkx2.5 transcription in this region.

GATA Factors Have Multiple Functions in Cardiovascular Development

From this work we conclude that expression of GATA factors in precardiac mesoderm is not sufficient to activate a myocardial differentiation program. Instead, the data indicate that GATA factor activity may need to be moderated for differentiation to proceed, consistent with the misexpression experiments using GATA-6 (Gove *et al.*, 1997) and providing indirect evidence that GATA factors in heart mesoderm define the boundaries of the cardiac morphogenetic field. This adds to a growing list of experimentally determined functions for GATA factors in the developing cardiovascular system, including development of cardiac-associated endoderm (Narita *et al.*, 1997, and our unpublished results), heart tube formation and morphogenesis (Jiang *et al.*, 1998; Kostetskii *et al.*, 1999; Kuo *et al.*, 1997; Molkentin *et al.*, 1997), cardiomyocyte proliferation (Gove *et al.*, 1997), cardiomyocyte differentiation (Grepin *et al.*, 1995, 1997), hypertrophic response (Molkentin *et al.*, 1998), and great vessel smooth muscle cell differentiation (Mano *et al.*, 1999; Suzuki *et al.*, 1996). In contrast to GATA factors in the hematopoietic system, which appear to control primarily cell proliferation and differentiation decisions within specific cell lineages, the cardiac-associated GATA factors function in multiple germ layers, cell types, and patterning processes. Defining the target genes for GATA factors that control patterning and morphogenetic programs provides an important future challenge.

ACKNOWLEDGMENTS

The authors thank the Woodland and DePinho labs for providing plasmids. This work was made possible by funding from the American Heart Association, of which T.E. is an Established Investigator. T.A.D. acknowledges support from the Medical Research Council of Canada, the National Sciences and Engineering Research Council, and the Heart and Stroke Foundation of Canada.

REFERENCES

Arceci, R. J., King, A. A. J., Simon, M. C., Orkin, S. H., and Wilson, D. B. (1993). Mouse GATA-4: A retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol. Cell Biol.* **13,** 2235–2246.

Bodmer, R. (1993). The gene tinman is required for specification of the heart and visceral muscles in Drosophila. *Development* **118,** 719–729.

Boylan, J. F., Luftkin, T., Achkar, C. C., Taneja, R., Chambon, P., and Gudas, L. J. (1995). Targeted disruption of retinoic acid receptor alpha (RAR alpha) and RAR gamma results in receptor-specific alterations in retinoic acid-mediated differentiation and retinoic acid metabolism. *Mol. Cell Biol.* **15,** 843–851.

Charron, F., and Nemer, M. (1999). GATA transcription factors and cardiac development. *Semin. Cell Dev. Biol.* **10,** 85–91.

Cleaver, O. B., Patterson, K. D., and Krieg, P. A. (1996). Overexpression of the tinman-related genes XNkx-2.5 and XNkx-2.3 in Xenopus embryos results in myocardial hyperplasia. *Development* **122,** 3549–3556.

Drysdale, T. A., Patterson, K. D., Saha, M., and Krieg, P. A. (1997). Retinoic acid can block differentiation of the myocardium after heart specification. *Dev. Biol.* **188,** 205–215.

Drysdale, T. A., Tonissen, K. F., Patterson, K. D., Crawford, M. J., and Krieg, P. A. (1994). *Xenopus* cardiac troponin I is an embryonic heart specific marker: Expression during abnormal heart morphogenesis. *Dev. Biol.* **165,** 432–441.

Durocher, D., Charron, F., Warren, R., Schwartz, R. J., and Nemer, M. (1997). The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J.* **16,** 5687–5696.

Durocher, D., and Nemer, M. (1998). Combinatorial interactions regulating cardiac transcription. *Dev. Genet.* **22,** 250–262.

Durston, A. J., Timmermans, J. P. M., Hage, W. J., Hendriks, H. F. J., de Vries, N. J., Heideveld, M., and Nieuwkoop, P. D. (1989). Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature* **340,** 140–144.

Ehrman, L. A., and Yutzey, K. E. (1999). Lack of regulation in the heart forming region of avian embryos. *Dev. Biol.* **207,** 163–175.

Evans, S. M., Yan, W., Murillo, M. P., Ponce, J., and Papalopulu, N. (1995). tinman, a Drosophila homeobox gene required for heart and visceral mesoderm specification, may be represented by a family of genes in vertebrates: XNkx-2.3, a second vertebrate homologue of tinman. *Development* **121,** 3889–3899.

Evans, T. (1997). Regulation of cardiac gene expression by GATA-4/5/6. *Trends Card. Med.* **7,** 75–83.

Evans, T., and Felsenfeld, G. (1991). Trans-activation of a globin promoter in non-erythroid cells. *Mol. Cell Biol.* **11,** 843–853.

Fu, Y., Yan, W., Mohun, T. J., and Evans, S. M. (1998). Vertebrate tinman homologues XNkx2-3 and XNkx2-5 are required for heart formation in a functionally redundant manner. *Development* **125,** 4439–4449.

Gao, X., Sedgwick, T., Shi, Y.-B., and Evans, T. (1998). Distinct functions are implicated for the GATA-4, -5, and -6 transcription factors in the regulation of intestine epithelial cell differentiation. *Mol. Cell. Biol.* **18,** 2901–2911.

Gove, C., Walmsley, M., Nijjar, S., Bertwistle, D., Guille, M., Partington, G., Bomford, A., and Patient, R. (1997). Overexpression of GATA-6 in *Xenopus* embryos blocks differentiation of heart precursors. *EMBO J.* **16,** 355–368.

Grepin, C., Dagnino, L., Robitaille, L., Haberstroh, L., Antakly, T., and Nemer, M. (1994). A hormone-encoding gene identifies a pathway for cardiac but not skeletal muscle gene transcription. *Mol. Cell Biol.* **14,** 3115–3129.

Grepin, C., Nemer, G., and Nemer, M. (1997). Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. *Development* **124,** 2387–2395.

Grepin, C., Robitaille, L., Antakly, T., and Nemer, M. (1995). Inhibition of transcription factor GATA-4 expression blocks in vitro cardiac muscle differentiation. *Mol. Cell Biol.* **15,** 4095–4102.

Grow, M. W., and Krieg, P. A. (1998). Tinman function is essential for vertebrate heart development: Elimination of cardiac differentiation by dominant inhibitory mutants of the tinman-related genes, XNkx2-3 and XNkx2-5. *Dev. Biol.* **204,** 187–196.

Gruber, P. J., Kubalak, S. W., Pexieder, T., Sucov, H. M., Evans, R. M., and Chien, K. R. (1996). RXRα deficiency confers genetic susceptibility for aortic sac, conotruncal, atrioventricular cushion, and ventricular muscle defects in mice. *J. Clin. Invest.* **98,** 1332–1343.

Harper, S. E., Qiu, Y., and Sharp, P. A. (1996). Sin3 corepressor function in Myc-induced transcription and transformation. *Proc. Natl. Acad. Sci. USA* **93,** 8536–8540.

Hoffman, C., and Eichele, G. (1994). Retinoids in development. *In* "The Retinoids: Biology, Chemistry, and Medicine" (M. B. Sporn, A. B. Roberts, and D. S. Goodman, Eds.), pp. 387–441. Raven Press, New York.

Hudson, C., Clements, D., Friday, R. V., Stott, D., and Woodland, H. R. (1997). Xsox17α and β mediate endoderm formation in *Xenopus. Cell* **91,** 397–405.

Ip, H. S., Wilson, D. B., Heikinheimo, M., Tang, Z., Ting, C.-N., Simon, M. C., Leiden, J. M., and Parmacek, M. S. (1994). The GATA-4 transcription factor transactivates the cardiac muscle-specific troponin C promoter-enhancer in nonmuscle cells. *Mol. Cell Biol.* **14,** 7517–7526.

Jiang, Y., and Evans, T. (1996). The Xenopus GATA-4/5/6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during embryogenesis. *Dev. Biol.* **174,** 258–270.

Jiang, Y., Tarzami, S., Burch, J. B. E., and Evans, T. (1998). Common role for each of the cGATA-4/5/6 genes in the regulation of cardiac morphogenesis. *Dev. Genet.* **22,** 263–277.

Kastner, P., Grondona, J. M., Mark, M., Gansmuller, A., LeMeur, M., Decimo, D., Vonesch, J. L., Dolle, P., and Chambon, P. (1994). Genetic analysis of RXRα developmental function: Convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell* **78,** 987–1003.

Kastner, P., Messaddeq, N., Mark, M., Wendling, O., Grondona, J. M., Ward, S., Ghyselinck, N., and Chambon, P. (1997). Vitamin A deficiency and mutations of RXRα, RXRβ, and RARα lead to early differentiation of embryonic ventricular cardiomyocytes. *Development* **124,** 4749–4758.

Kelley, C., Blumberg, H., Zon, L. I., and Evans, T. (1993). GATA-4 is a novel transcription factor expressed in endocardium of the developing heart. *Development* **118,** 817–827.

Kostetskii, I., Jiang, Y., Kostetskaia, E., Yuan, S., Evans, T., and Zile, M. (1999). Retinoid signaling required for normal heart development regulates GATA-4 in a pathway distinct from cardiomyocyte differentiation. *Dev. Biol.* **206,** 206–218.

Koutsourakis, M., Langeveld, A., Patient, R., Beddington, R., and Grosveld, F. (1999). The transcription factor GATA6 is essential for early extraembryonic development. *Development* **126,** 723–732.

Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C., and Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev.* **11,** 1048–1060.

Laverriere, A. C., NacNeill, C., Mueller, C., Poelmann, R. E., Burch, J. B. E., and Evans, T. (1994). GATA-4/5/6: A subfamily of three transcription factors expressed in developing heart and gut. *J. Biol. Chem.* **269,** 23177–23184.

Lee, R. Y., Luo, J., Evans, R. M., Giguere, V., and Sucov, H. M. (1997). Compartment-selective sensitivity of cardiovascular

morphogenesis to combinations of retinoic acid receptor gene mutations. *Circ. Res.* **80,** 757–764.

Lien, C. L., Wu, C., Mercer, B., Webb, R., Richardson, J. A., and Olson, E. N. (1999). Control of early cardiac-specific transcription of Nkx2-5 by a GATA-dependent enhancer. *Development* **126,** 75–84.

Lu, J. R., McKinsey, T. A., Xu, H., Wang, D. Z., Richardson, J. A., and Olson, E. N. (1999). FOG-2, a heart- and brain-enriched cofactor for GATA transcription factors. *Mol. Cell Biol.* **19,** 4495–4502.

Luo, J., Sucov, H. M., Bader, J.-A., Evans, R. M., and Giguere, V. (1996). Compound mutants for retinoic acid receptor (RAR) β and RARα1 reveal developmental functions for multiple RARβ isoforms. *Mech. Dev.* **55,** 33–44.

Lyons, G. E. (1996). Vertebrate heart development. *Curr. Opin. Gen. Dev.* **6,** 454–460.

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L., and Harvey, R. P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeobox gene Nkx2-5. *Genes Dev.* **9,** 1654–1666.

Mano, T., Luo, Z., Malendowicz, S. L., Evans, T., and Walsh, K. (1999). Reversal of GATA-6 downregulation promotes muscle differentiation and inhibits intimal hyperplasia in balloon-injured rat carotid artery. *Circ. Res.* **84,** 647–654.

Mohun, T., and Sparrow, D. (1997). Early steps in vertebrate cardiogenesis. *Curr. Opin. Gen. Dev.* **7,** 628–633.

Molkentin, J. D., Kalvakolanu, D. V., and Markham, B. E. (1994). Transcription factor GATA-4 regulates cardiac muscle-specific expression of the α-myosin heavy-chain gene. *Mol. Cell Biol.* **14,** 4947–4957.

Molkentin, J. D., Lin, Q., Duncan, S. A., and Olson, E. N. (1997). Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev.* **11,** 1061–1072.

Molkentin, J. D., Lu, J.-R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1998). A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* **93,** 215–228.

Moody, S. A. (1987). Fates of the blastomeres of the 32-cell stage *Xenopus* embryo. *Dev. Biol.* **122,** 300–319.

Morrisey, E. E., Tang, Z., Sigrist, K., Lu, M. M., Jiang, F., Ip, H. S., and Parmacek, M. S. (1998). GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev.* **12,** 3579–3590.

Narita, N., Bielinska, M., and Wilson, D. B. (1997). Wild-type endoderm abrogates the ventral developmental defects associated with GATA-4 deficiency in the mouse. *Dev. Biol.* **189,** 270–274.

Nascone, N., and Mercola, M. (1995). An inductive role for the endoderm in *Xenopus* cardiogenesis. *Development* **121,** 515–523.

Newman, C. S., and Krieg, P. A. (1998). Tinman-related genes expressed during heart development in *Xenopus*. *Dev. Genet.* **22,** 230–238.

Nieuwkoop, P. D., and Faber, J. (1967). "Normal Table of *Xenopus laevis* (Daudin)." North-Holland, Amsterdam.

Peng, H. B. (1991). Appendix A. Solutions and protocols. *In "Xenopus laevis:* Practical Uses in Cell and Molecular Biology" (B. K. Kay and H. B. Peng, Eds.), Vol. 36, pp. 661–662. Academic Press, San Diego.

Sater, A. K., and Jacobson, A. G. (1989). The specification of heart mesoderm occurs during gastrulation in Xenopus laevis. *Development* **105,** 821–830.

Sater, A. K., and Jacobson, A. G. (1990a). The restriction of the heart morphogenetic field in *Xenopus laevis*. *Dev. Biol.* **140,** 328–336.

Sater, A. K., and Jacobson, A. G. (1990b). The role of the dorsal lip in the induction of heart mesoderm in *Xenopus laevis*. *Development* **108,** 461–470.

Schott, J.-J., Benson, D. W., Basson, C. T., Pease, W., Silberbach, G. M., Moak, J. P., Maron, B. J., Seidman, C. E., and Seidman, J. G. (1998). Congenital heart disease caused by mutations in the transcription factor NKX2–5. *Science* **281,** 108–111.

Schreiber-Agus, N., Chin, L., Chen, K., Torres, R., Rao, G., Guida, P., Skoultchi, A. I., and DePinho, R. A. (1995). An amino-terminal domain of Mxi1 mediates anti-myc oncogenic activity and interacts with a homolog of the yeast transcriptional repressor SIN3. *Cell* **80,** 777–786.

Schultheiss, T. M., Burch, J. B. E., and Lassar, A. B. (1997). A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes Dev.* **11,** 451–462.

Searcy, R. D., Vincent, E. B., Liberatore, C. M., and Yutzey, K. E. (1998). A GATA-dependent Nkx-2.5 regulatory element activates early cardiac gene expression in transgenic mice. *Development* **125,** 4461–4470.

Sepulveda, J. L., Belaguli, N., Nigam, V., Chen, C. Y., Nemer, M., and Schwartz, R. J. (1998). GATA-4 and Nkx-2.5 coactivate Nkx-2 DNA binding targets: Role for regulating early cardiac gene expression. *Mol. Cell Biol.* **18,** 3405–3415.

Serbedzija, G. N., Chen, J.-N., and Fishman, M. C. (1998). Regulation in the heart field of zebrafish. *Development* **125,** 1095–1101.

Sive, H. L., Draper, B. W., Harland, R. M., and Weintraub, H. (1990). Identification of a retinoic acid-sensitive period during axis formation in *Xenopus laevis*. *Genes Dev.* **4,** 932–942.

Smith, S. M., and Dickman, E. D. (1997). New insights into retinoid signaling in cardiac development and physiology. *Trends Card. Med.* **7,** 324–329.

Soudais, C., Bielinska, M., Heikinheimo, M., MacArthur, C. A., Narita, N., Saffitz, J. E., Simon, M. C., Leiden, J. M., and Wilson, D. B. (1995). Targeted mutagenesis of the transcription factor GATA-4 gene in mouse embryonic stem cells disrupts visceral endoderm differentiation in vitro. *Development* **121,** 3877–3888.

Sucov, H. M., Dyson, E., Gumeringer, C. L., Price, J., Chien, K. R., and Evans, R. M. (1994). RXRα mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. *Genes Dev.* **8,** 1007–1018.

Suzuki, E., Evans, T., Lowry, J., Truong, L., Bell, D. W., Testa, J. R., and Walsh, K. (1996). The human GATA-6 gene: Structure, chromosomal location and regulation of expression by tissue-specific and mitogen-responsive signals. *Genomics* **38,** 283–290.

Svensson, E. C., Tufts, R. L., Polk, C. E., and Leiden, J. M. (1999). Molecular cloning of FOG-2: A modulator of transcription factor GATA-4 in cardiomyocytes. *Proc. Natl. Acad. Sci. USA* **96,** 956–961.

Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N., and Izumo, S. (1999). The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. *Development* **126,** 1269–1280.

Tanaka, M., Kasahara, H., Bartunkova, S., Schinke, M., Komuro, I., Inagaki, H., Lee, Y., Lyons, G. E., and Izumo, S. (1998). Vertebrate

homologs of tinman and bagpipe: Roles of the homeobox genes in cardiovascular development. *Dev. Genet.* **22,** 239–249.

Tonissen, K. F., Drysdale, T. A., Lints, T., Harvey, R. P., and Krieg, P. A. (1994). Xnkx-2.5, a *Xenopus* gene related to Nkx-2.5 and *tinman:* Evidence for a conserved role in cardiac development. *Dev. Biol.* **162,** 325–328.

Yang, H.-Y., and Evans, T. (1992). Distinct roles for the two cGATA-1 finger domains. *Mol. Cell Biol.* **12,** 4562–4570.

Yutzey, K. E., Gannon, M., and Bader, D. (1995). Diversification of cardiomyogenic cell lineages in vitro. *Dev. Biol.* **170,** 531–541.

Yutzey, K. E., Rhee, J. T., and Bader, D. (1994). Expression of the atrial-specific myosin heavy chain AMHC1 and the establishment of anteroposterior polarity in the developing chicken heart. *Development* **120,** 871–883.

Zile, M. (1998). Vitamin A and embryonic development: An overview. *J. Nutr.* **128,** 455S–458S.

Received for publication June 14, 1999
Revised August 24, 1999
Accepted August 26, 1999